Gene engineering and biotechnology of higher plants I

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Biotechnology of Drugs 2024

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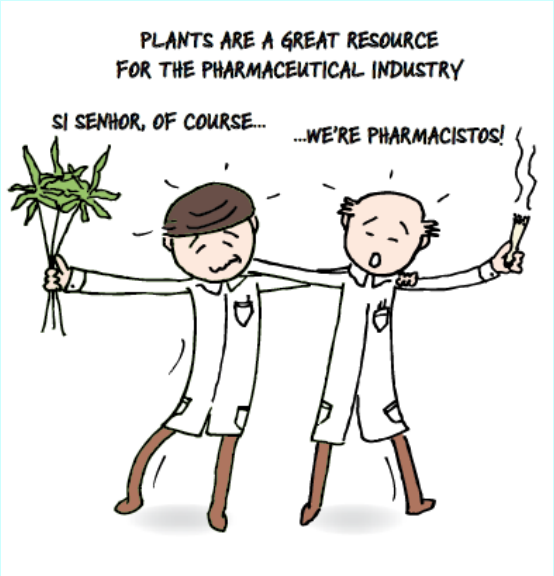
1. Structure of plant DNA
2. What can be manipulated in plants
3. Genetic transformation of plants
4. Transformation by *Agrobacterium*
5. Ti/Ri plasmids, T-DNA
6. Transformation by “naked“ DNA
7. Protoplast transformation
8. Plant tissue cultures

Why to produce GM plants?

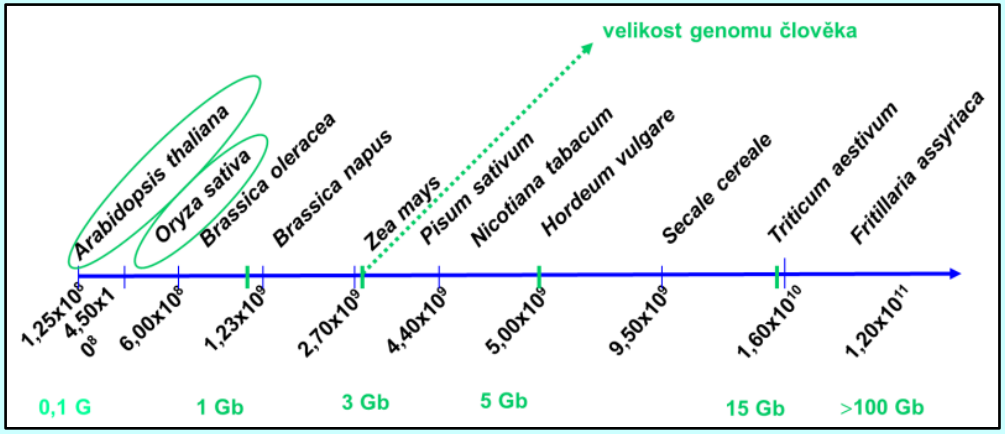
- Biomass and biofuel production
- Extending shelf life
- Increased resistance to stress and pests
- Improved taste



- Higher nutritional value, nutraceuticals, functional food (more vitamins, etc.)
- Disease prevention/treatment products (protein production, secondary metabolites, etc.)

Plant genomes

- Significant size of nuclear DNA
- Frequent polyploidy
- Large numbers of chromosomes, 2n = 4 → 2n = 600
- Numerous duplications
- Numerous transpositions
- Low frequency of homologous recombination of nuclear DNA



Example – genes of *Arabidopsis thaliana*

	Nucleus	Plastid	Mitochondrion
Size	125 Mb	154 kb	367 kb
Duplication	60 %	17 %	10 %
Structural genes	25 498	79	58
Density (kb per 1 gene)	4.5	1	6.25
Complex genes	79 %	18,4	12 %
Transposons	10 %		4 %

rDNA in plant genome

Nuclear DNA

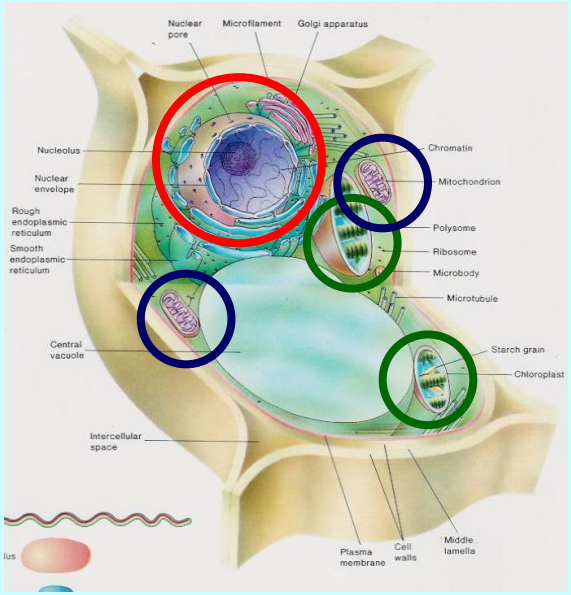
- 25S - 28S rRNA; 5.8S rRNA; 5S rRNA
- 17S - 18S rRNA

Chloroplast DNA

- 23S rRNA; 4.5S rRNA; 5S rRNA
- 16S rRNA

Mitochondrial DNA

- 23S rRNA; 4.5S rRNA; 5S rRNA
- 18S rRNA



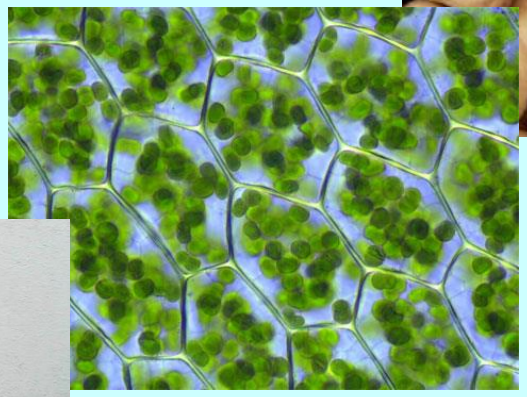
What is worth cloning in plants?

Genes, which

- enable production of new species
- have better features
- have new features

The most frequently cloned genes

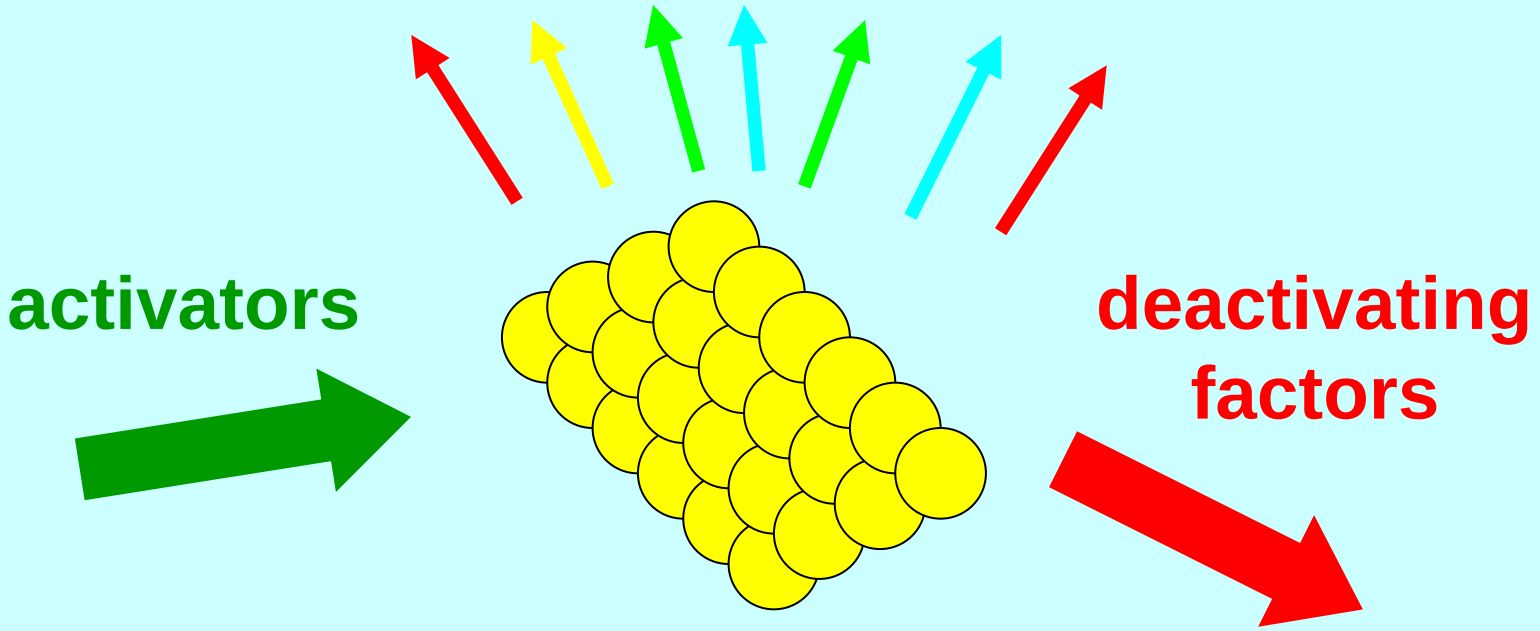
- Storage proteins of seeds and tubers
- Enzymes of photosynthesis
- Stress proteins



Main factors of gene regulation

Different reactions of individual genes

Complex interactions = more factors influencing a specific gene



ten thousands of genes

Factors of gene regulation

- **Work on all levels of gene expression regulation**
- **Work on all types of regulatory sequences (promoters, activators, silencers, attenuators)**
- **Depend on ontogenesis**

They are site and time specific

Factors of gene regulation

Only thorough knowledge of plant biochemistry, physiology and cell morphology enable rational design of recombinant plants

Factors of gene regulation:

Internal

External

Internal factors of gene regulation

1. **Organ and cell specific regulation (connected with ontogenesis)**

2. **Phytohormones regulation**
   - **auxins, cytokines, gibberellins, abscise acid and ethylene**

     - level of transcription and higher levels

     - modify regulatory proteins

   - **Reaction to phytohormones changes during the ontogenesis**

We try to influence the internal factors of regulation genetic transformation of plants

External factors of gene regulation

1. Regulation by light
2. Regulation by stress factors
3. Regulation by circadian rhythms
4. Regulation by length of day
5. Regulation by specific substrates
6. Regulation by interactions with symbiotic organisms

Genes are active based on a specific stimulus - use of specific promoters

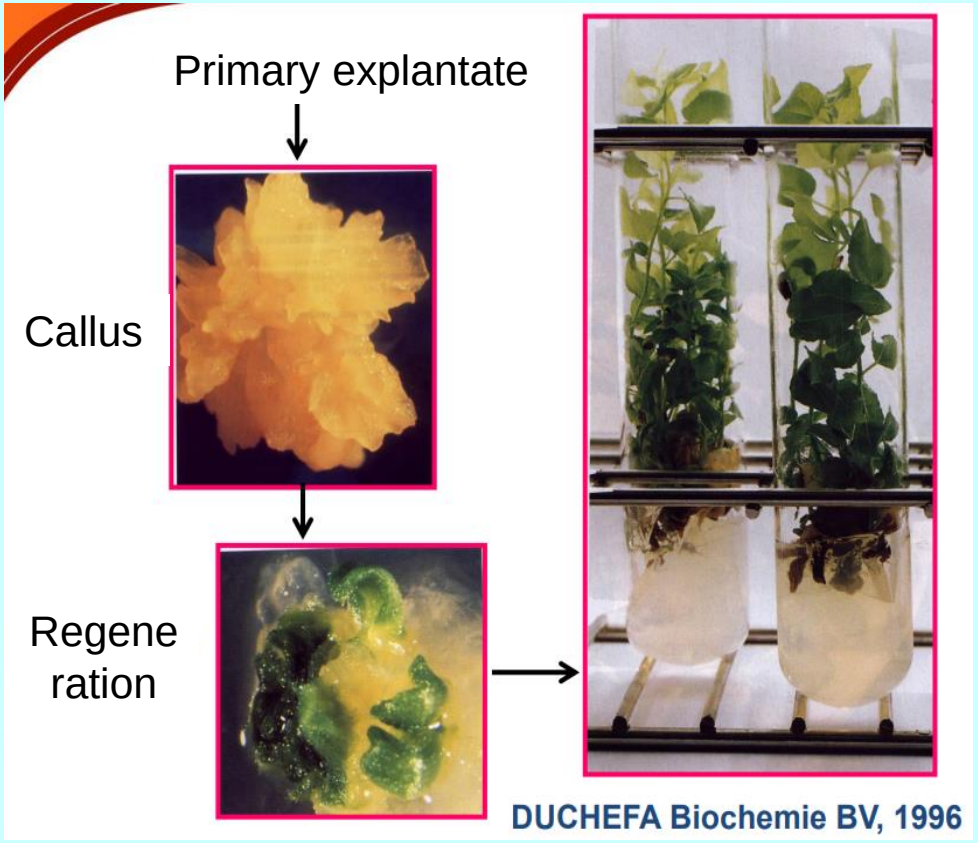
Scientists can influence the internal factors of gene regulation by genetic transformation of plants

Genetic transformation of plants

- **Transfer of foreign DNA into plant cells (protoplasts)**
- **Its integration into plant genome (nuclear or cytoplasmic)**
- **Expression of foreign DNA in a plant**
- **Ensuring mitotic and meiotic heredity of transferred genetic material**

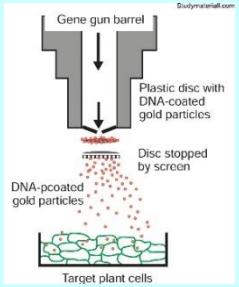
Totipotency

Each plant cell contains complete genetic information, which is necessary for the development of the whole organism



Steps in plant transformation

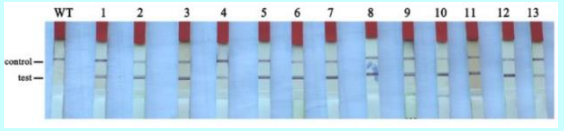
1. Preparation of DNA



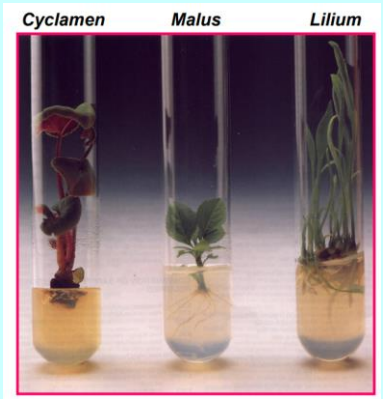
2. Transfer of DNA into a plant

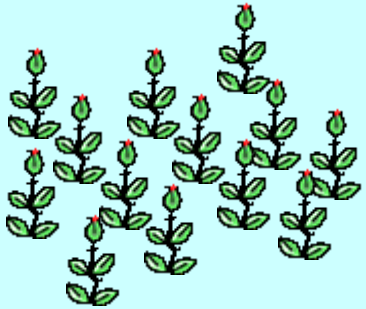
3. Screening and selection at the level of expression

4. Identification of a transgenic plant



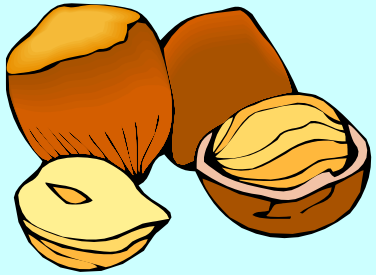
5. Plant regeneration





1. Isolation of plant DNA

- Complete and quick destruction of plant material
- Hard versus soft tissues, quickly growing sprouts



- Cell wall mechanically or by cellulolytic enzymes
- Cell membrane by detergents
- Separation of proteins and NAs by precipitation, affinity chromatography

2. Transfer of DNA into a plant

A. Methods of the DNA transfer

I. Transfer induced by infection by *Agrobacterium* using Ti and Ri plasmids

II. Direct transformation of DNA

a Bombarding by micro particles

b. Protoplasts transformation by electroporation or by polyethylene glycol

B. Cells, tissues and organs suitable for transformation

I. Morphologically competent cells

II. The whole plants

Vectors

Typical bacterial plasmids

They contain plant expression cassettes

Each cassette consists of

- enhancer/promoter (transcription control)

- segment of DNA, which is transcribed into mRNA

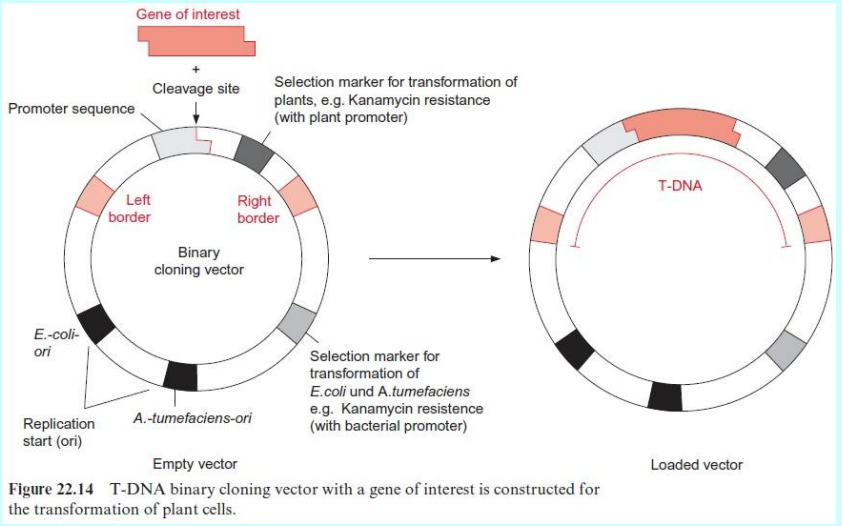


Figure 22.14 T-DNA binary cloning vector with a gene of interest is constructed for the transformation of plant cells.

https://www.brainkart.com/article/Ti-Plasmids-are-used-as-transformation-vectors_21529/

Expression cassette in a plant

enhancer/promoter	leader sequence 5'-UTR	coding sequence	terminator AAA/3'-UTR

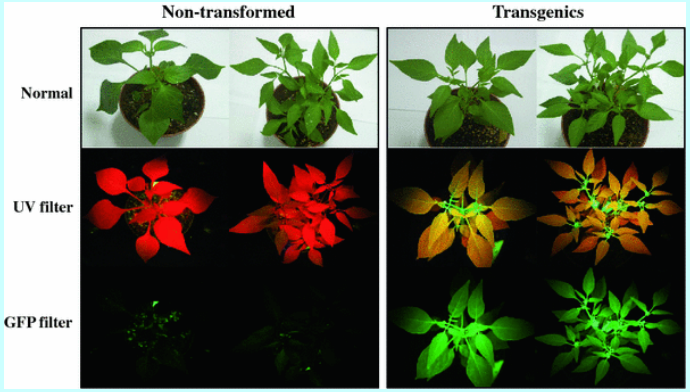
Selectable and reporter proteins

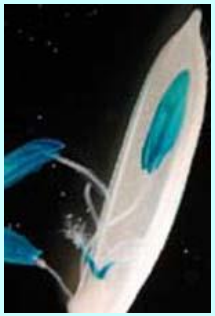
Selectable

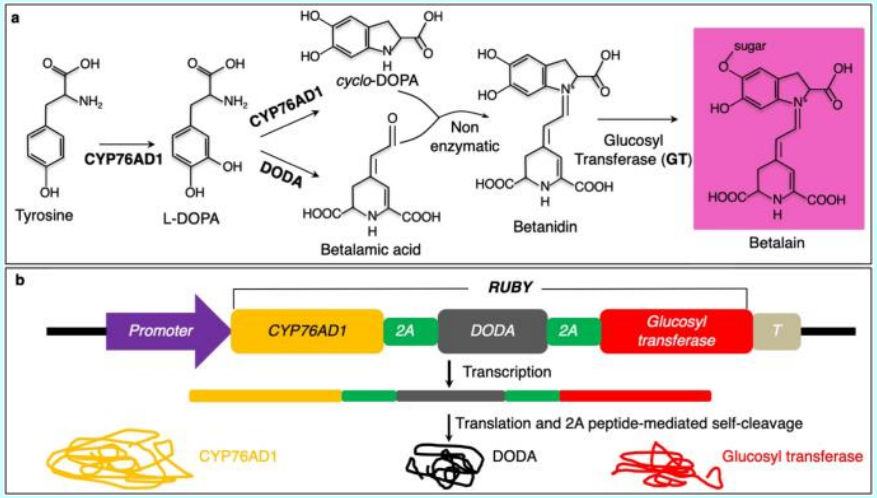
- ***NPT*** (neomycin phosphotransferase)
- ***HPT*** (hygromycin phosphotransferase)
- ***BAR*** (resistance to herbicide phosphonotricine)

Reporter (signal)

- ***GUS*** (β-glucuronidase) – substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc)
- *GFP* – fluorescent protein
- Creating a colour product (e.g. *RUBY*)



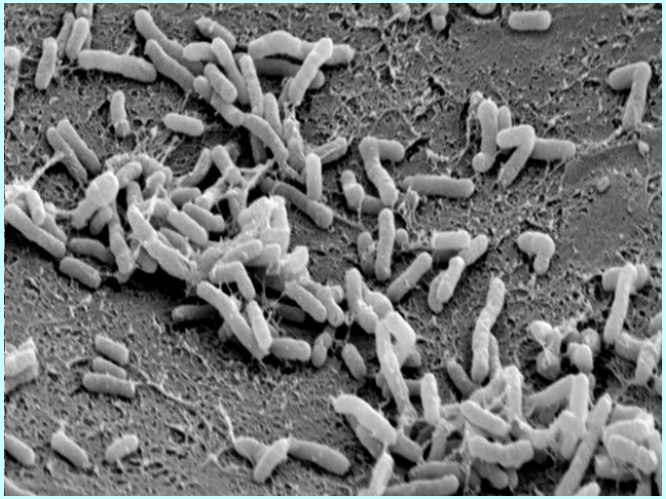




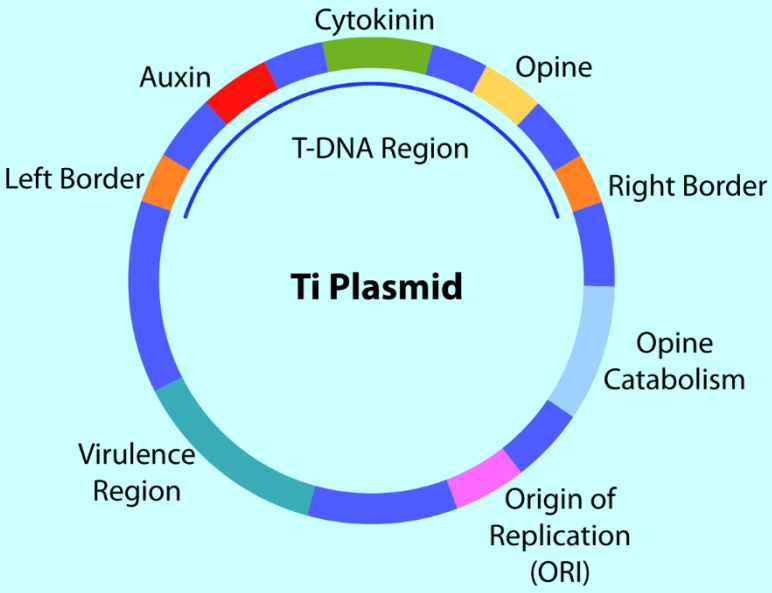
https://doi.org/10.1038/s41438-020-00390-1

Transformation by *Agrobacterium*

- **Soil bacterium family *Rhizobiaceae***
- **Component of root nodules of Leguminoses**
- **Only the genus *Agrobacterium* is able to transfer DNA by large plasmid molecules**



Ti-plasmids, Ri-plasmids



- **Ti-plasmid induces <u>crown galls</u> between the root and the stem**
- **Ri-plasmid induces <u>hairy roots</u>**
- **Both phenomena occur after the integration of T-DNA into the genome (only dicotyledonous plants)**

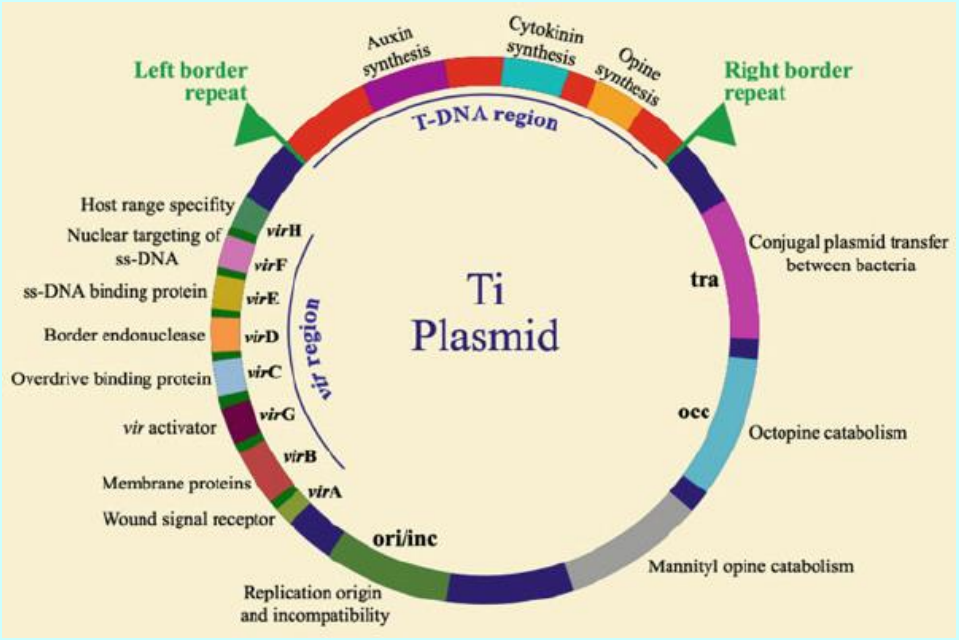
T-DNA

T-DNA is a region on Ti-plasmid and Ri-plasmid which integrates into nuclear DNA of plant cells

- **natural host of Ti-plasmid = *A. tumefaciens***
- **natural host of Ri-plasmid = *A. rhizogenes***

Genes on T-DNA

- **Genes for synthesis of auxins, gibberellins and cytokines**
- **Genes for synthesis of tumour specific compounds, opines**



https://www.thebiomics.com/notes/applied-biology/agrobacterium-tumefaciens-mediated-transformation.html

The rest of plasmid

- Vir genes - important for virulence and T-DNA transfer
- opines catabolism genes
- ori region
- tra region - for conjugation

Genes for auxins and giberellins

- **Cause differentiation of plant cells**
- **Transformed tissues grow as non-differentiated tumours**
- **In *in vitro* conditions the transformed tissues grow permanently without growth stimulators**
- **New shoots can be differentiated from transformed tissues - transformed and also non-transformed**

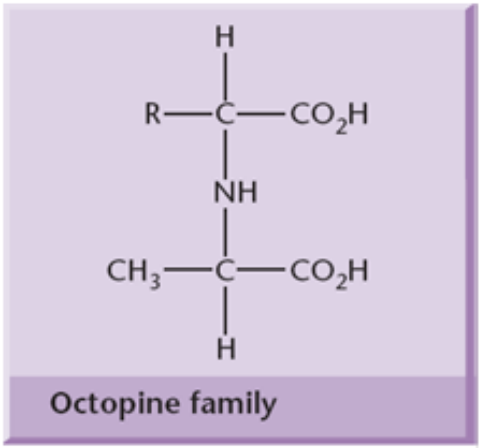
Opines – tumour specific compounds

- **Opines are source of carbon, nitrogen and energy for bacteria, which caused transformation**
- **Opines = derivatives of basic amino acids - Arg, Lys**

Opines of octopine type = AA + pyruvate

Opines of nopaline type = AA + α-oxoglutarate

Opines – structural features



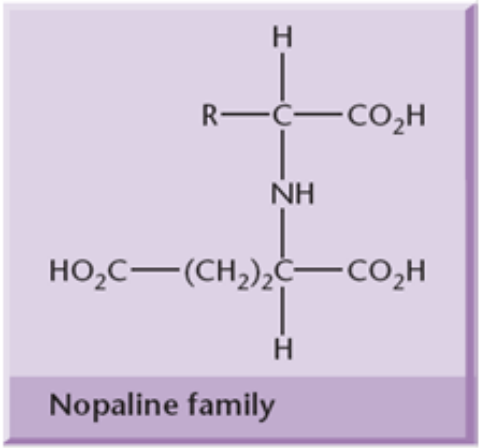


Figure 4: General structures of some opines

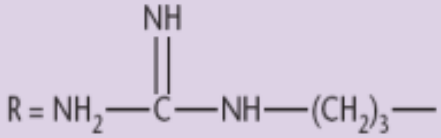
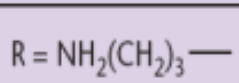
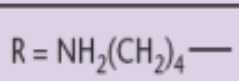
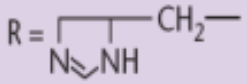
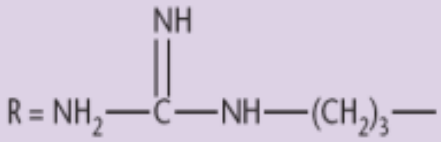
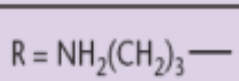
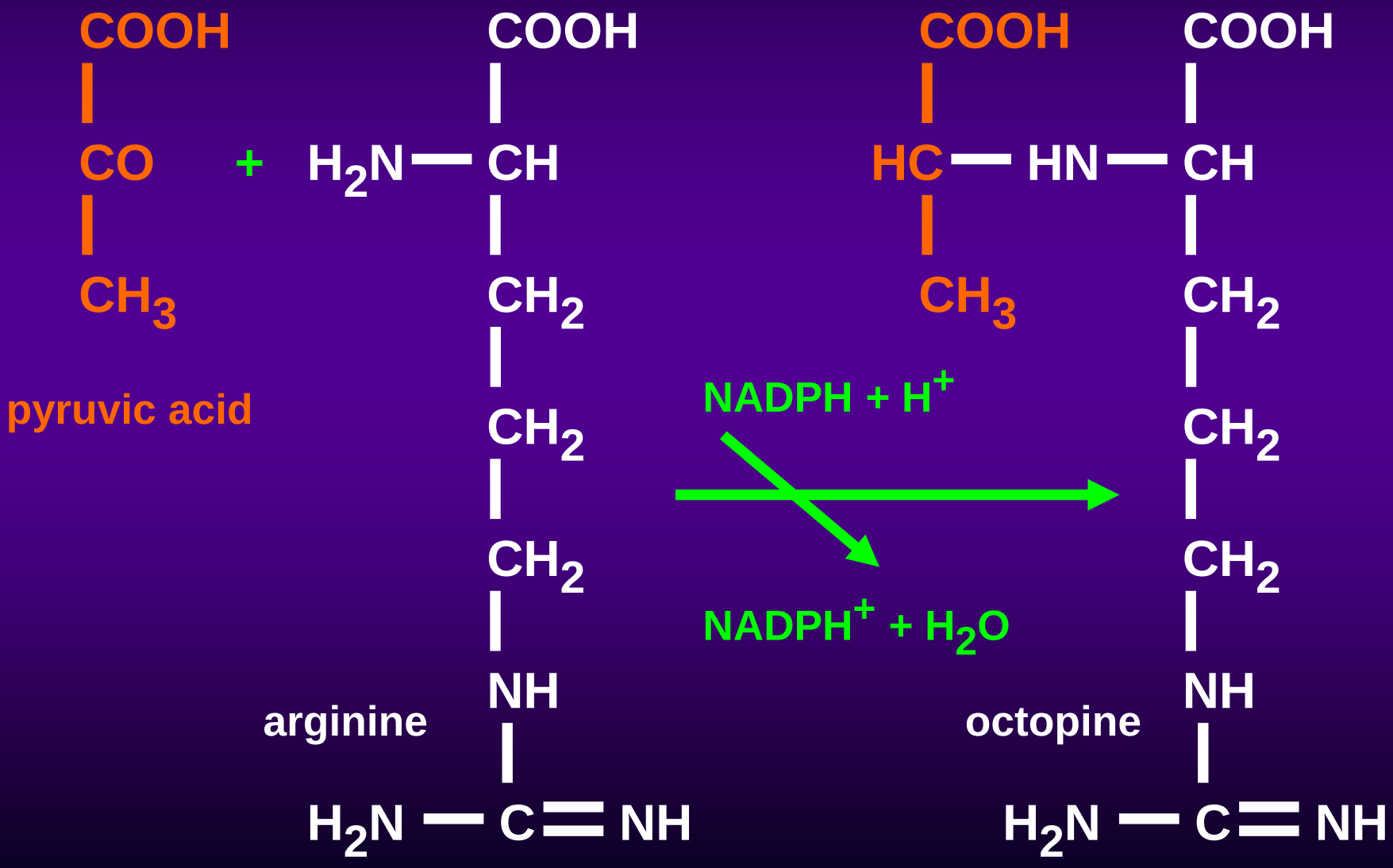
Octopine	
Octopinic acid	
Lysopine	
Histopine	
Nopaline	
Nopalinic acid	

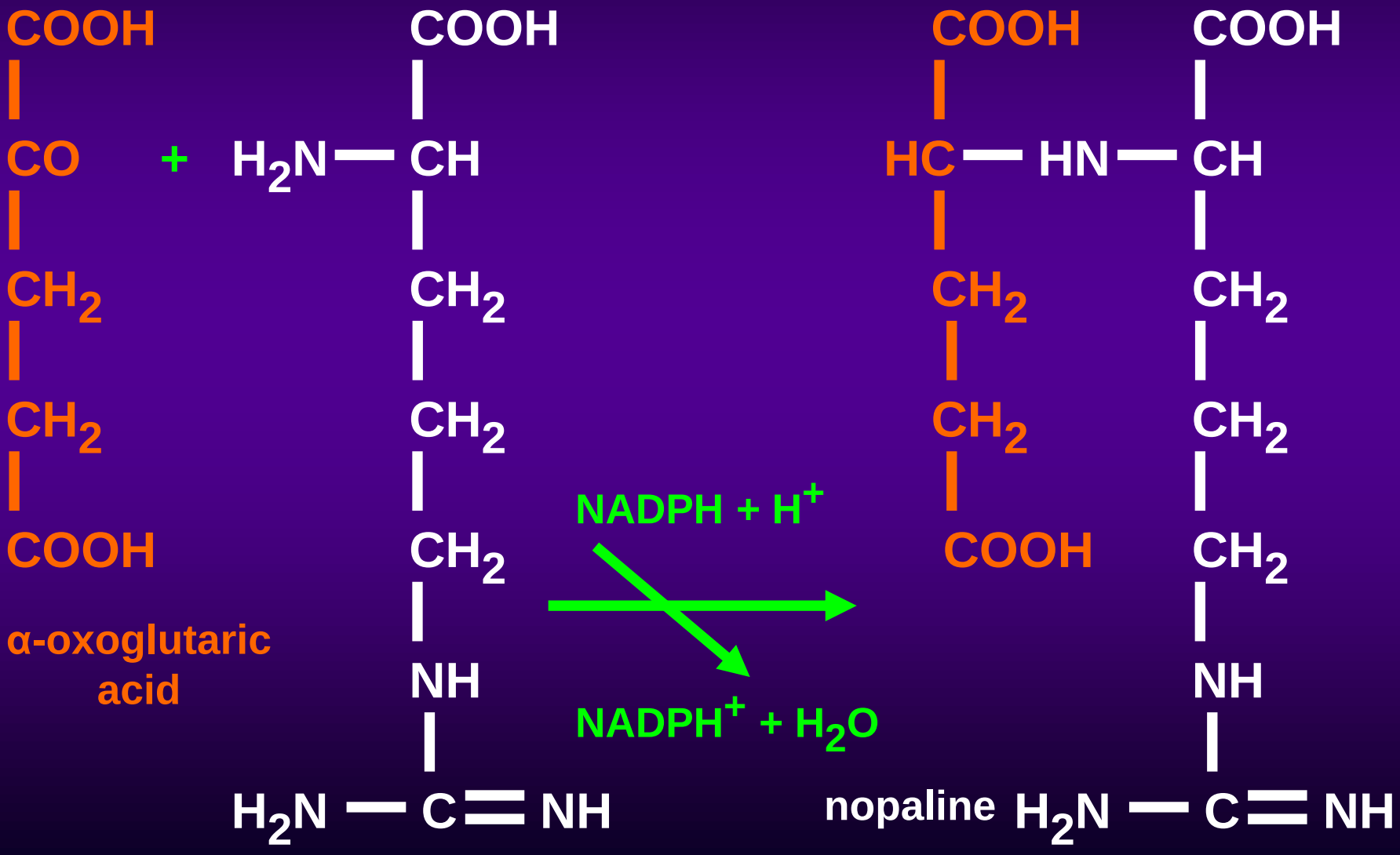
Figure 5: Structures of some opines

https://www.thebiomics.com/notes/applied-biology/agrobacterium-tumefaciens-mediated-transformation.html

Octopine structure



Nopaline structure

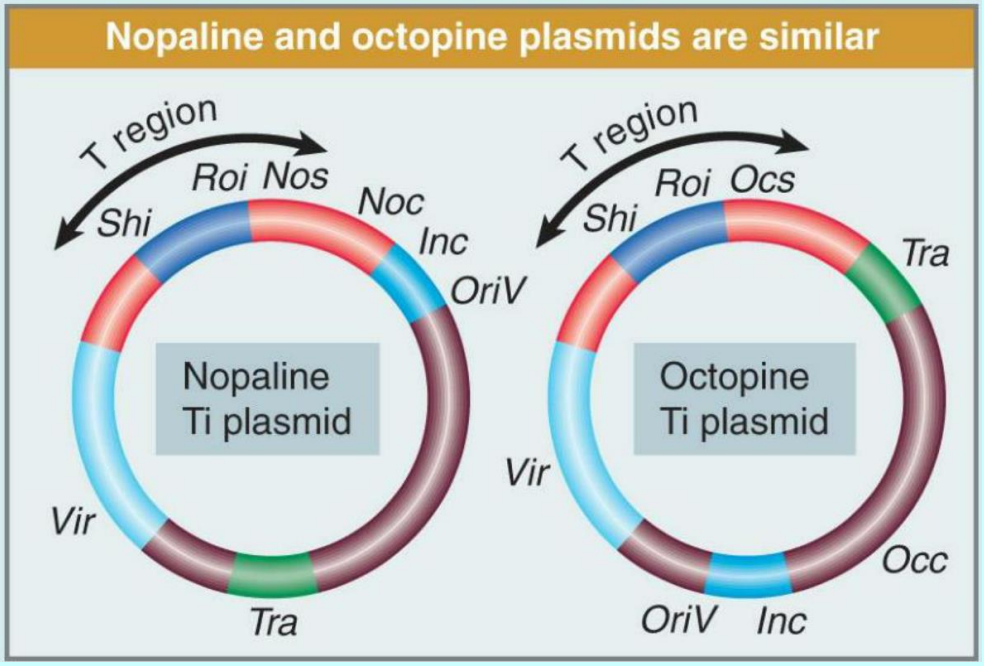


Genes and plasmids for opines

- **Synthesis of opines = genes on T-DNA**
- **Opines catabolism = genes in different part of Ti-plasmid (active only in bacteria)**

- **Ti-plasmid of octopine type**
  - bears genes for octopine synthesis
- **Ti-plasmid of nopaline type**
  - bears genes for nopaline synthesis

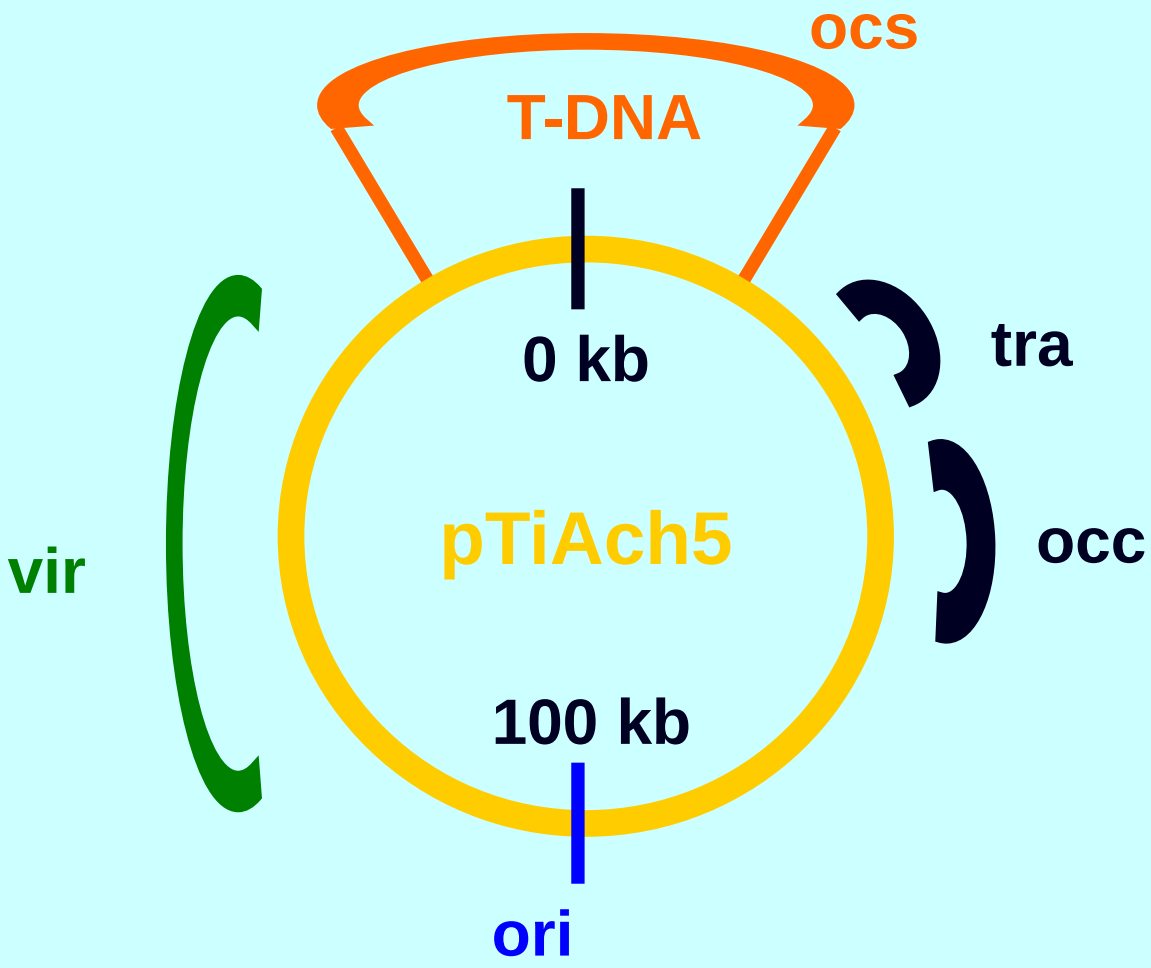
Structure of Ti/Ri plasmids



Ti genes function in bacteria and in plants

Locus	Function	Ti Plasmid
vir	DNA transfer into plant	all
shi	shoot induction	all
roi	root induction	all
nos	nopaline synthesis	nopaline
noc	nopaline catabolism	nopaline
ocs	octopine synthesis	octopine
occ	octopine catabolism	octopine
tra	bacterial transfer genes	all
Inc	incompatibility genes	all
oriV	origin for replication	all

Ti-plasmid of octopine type



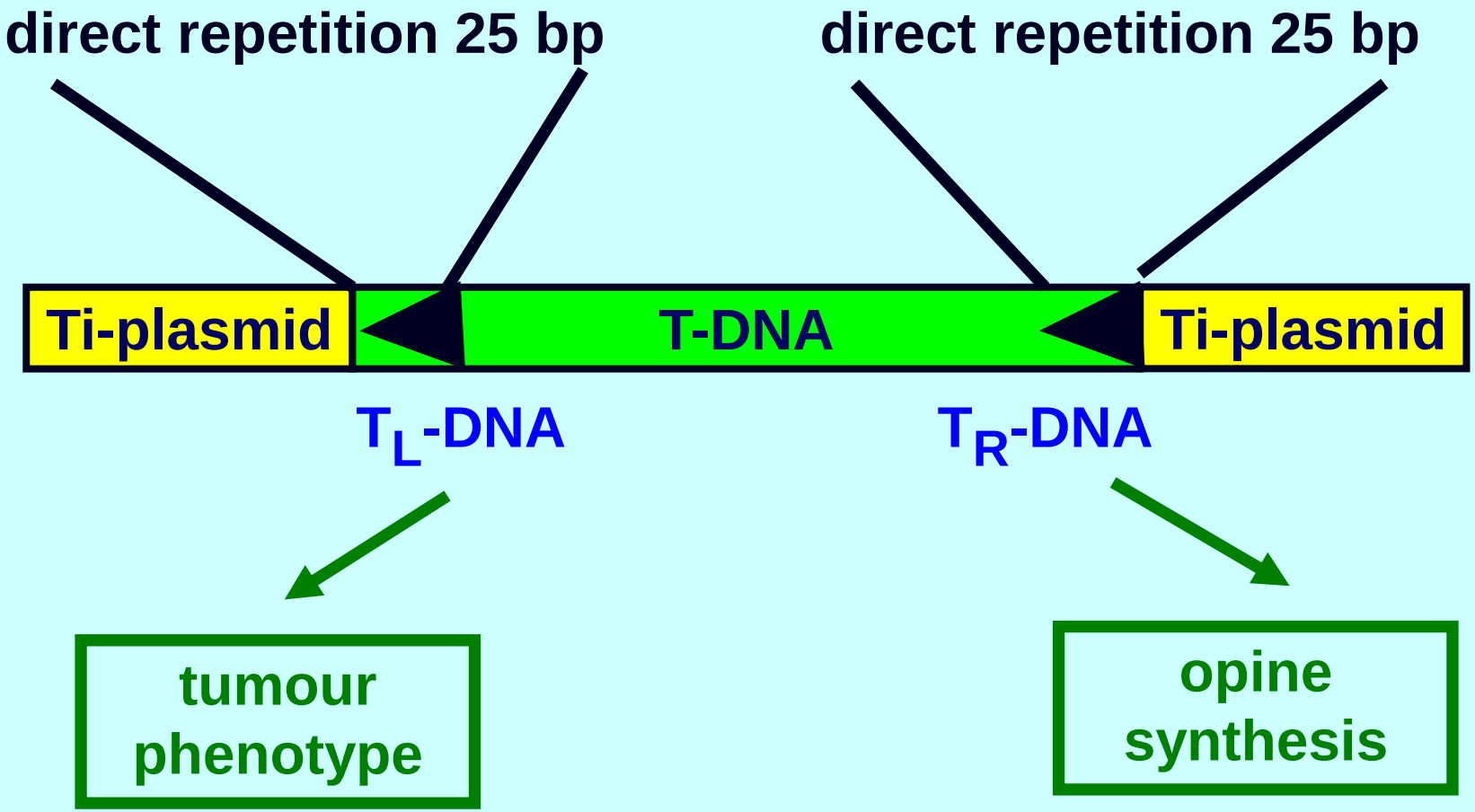
ocs – gene for octopine synthase, vir – genes of virulence

occ – genes of octopine catabolism

tra – genes for plasmid transformation by conjugation

T-DNA of octopine plasmids

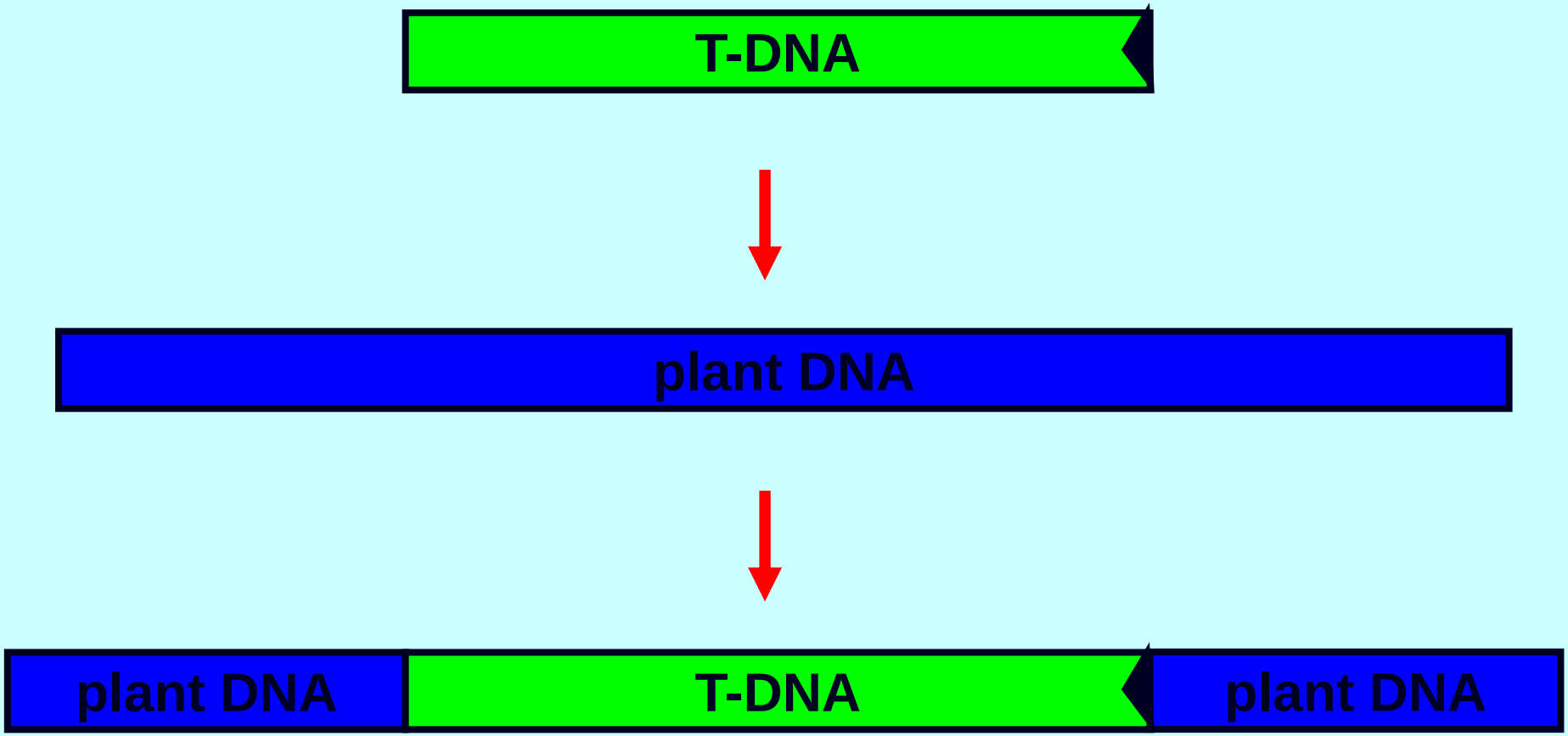
CGGCAGGATATATTCAATTGTAAAT



T-DNA of nopaline plasmids

- compact structure, non-defined left and right half
- surrounded as a whole by direct repeats on left and right end

T-DNA integration into genome



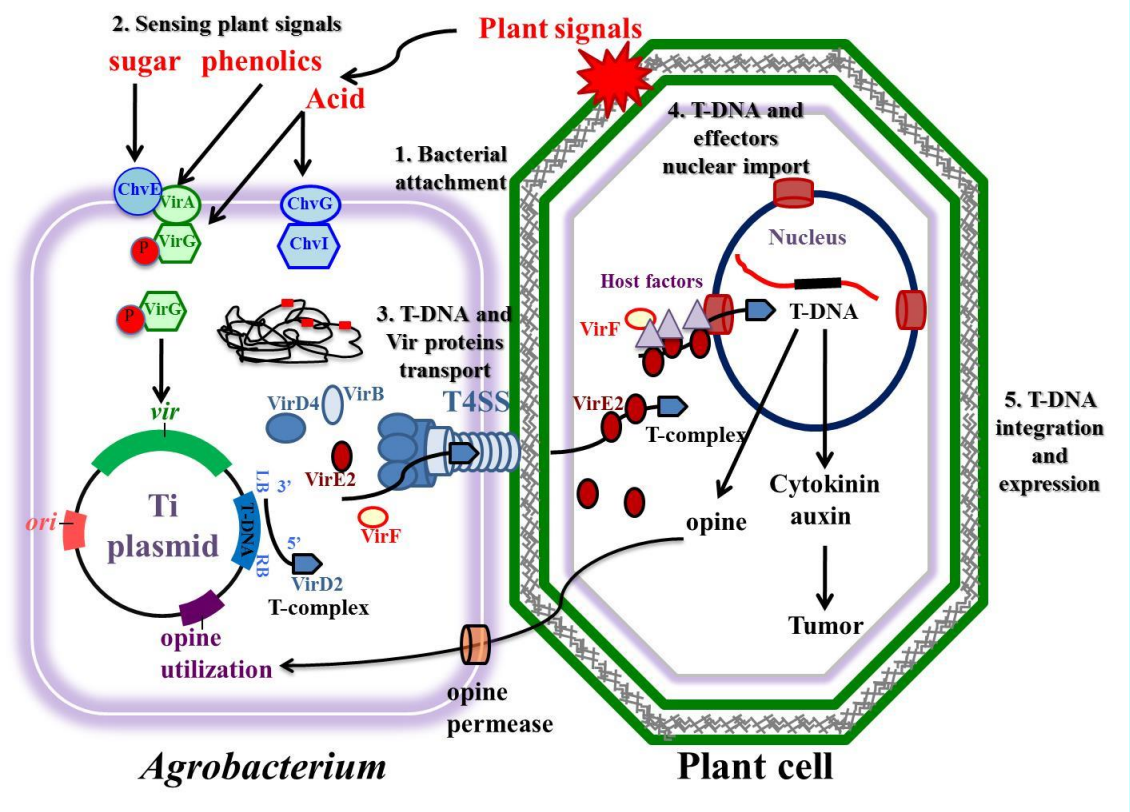
Genes of virulence

Located on Ti-plasmid outside T-DNA

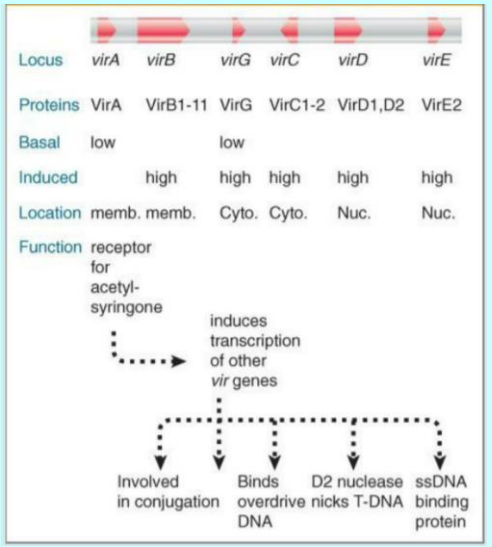
- **Their products recognize signal molecules of injured plants**
- **Code specific endonuclease, which digest boundary sequences of T-DNA**
- **Their products take part in the transfer of T-DNA into plant cells and its integration into chromosome**

Genes of virulence, which enable binding of Agrobacterium on plant cells during infection, are located on bacterial chromosome

Function of Vir proteins



https://doi.org/10.1199/tab.0186



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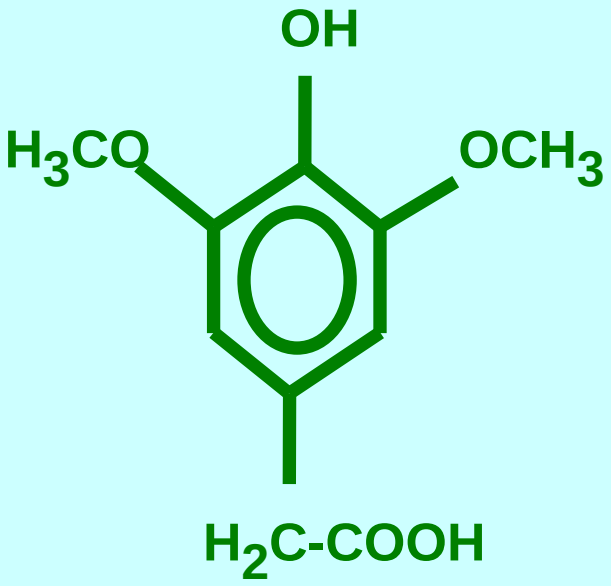
Virulence genes (ViR)

Six essential operons (*vir A, vir B, vir C, vir D, vir E, virG*) and two non-essential (*virF, virH*)

Vir Gene	Function
Vir A	A receptor activated by phenolic compounds, autophosphorylated and inturn phosphorylates VirG
VirG	VirG is Transcriptial factor necessary for expression of other virulence genes.
VirD2	Endonuclease; cuts T-DNA at right border to initiate T-strand synthesis and also it has an important role in integration of T DNA into host chromosomal DNA
Vir D1	Topiosomerase; Helps Vir D2 to recognise and cleave within the 25bp RB sequence
VirD4	VirD4 is the ATP-dependent linkage of protein complex necessary for T-DNA translocation
Vir C1	Promote high efficiency T-strand Synthesis; involved in spatial localization of the T-complex to the cell poles.
Vir E2	Binds to T-strand protecting it from nuclease attack, and intercalates with lipids to form channels in the plant membranes through which the T-complex passes.
Vir E1	Acts as a chaperone which stabilizes Vir E2 in the *Agrobacterium*.
Vir B & Vir D4	Assemble into a secretion system which spans the inner and outer bacterial membranes. Required for Export of the T-complex and Vir E2 into the plant cell

- *The bacterial infection and compatibility depends upon bacterial gene products such as Chv-A, B and C and Exo-C.*

Induction of genes of virulence I



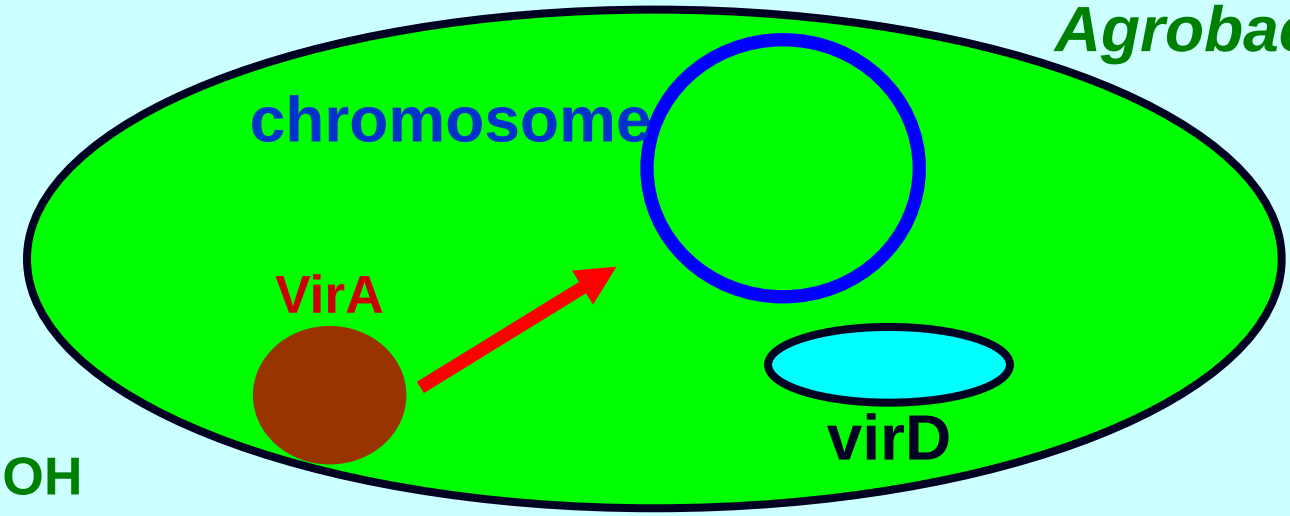
acetosyringone
(4-acetyl-2,6-dimetoxyphenol)

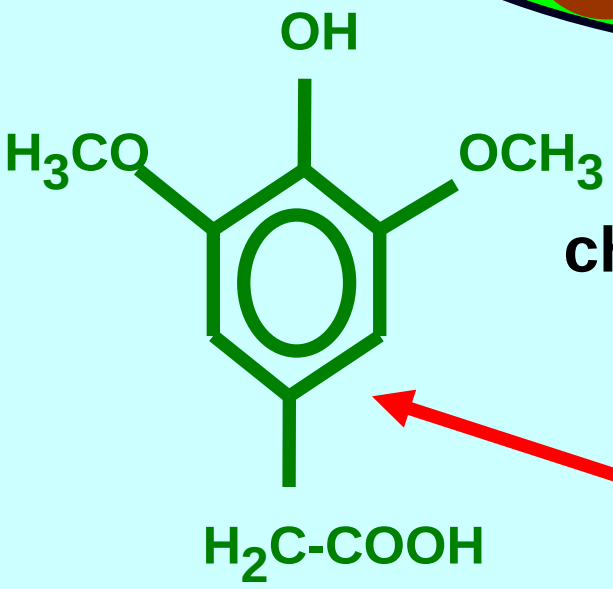
Injuring of plant cell

Production of phenol compounds

Induction of genes of virulence

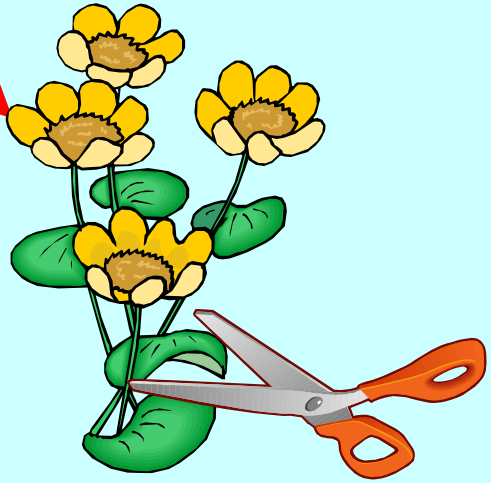
Induction of genes of virulence II





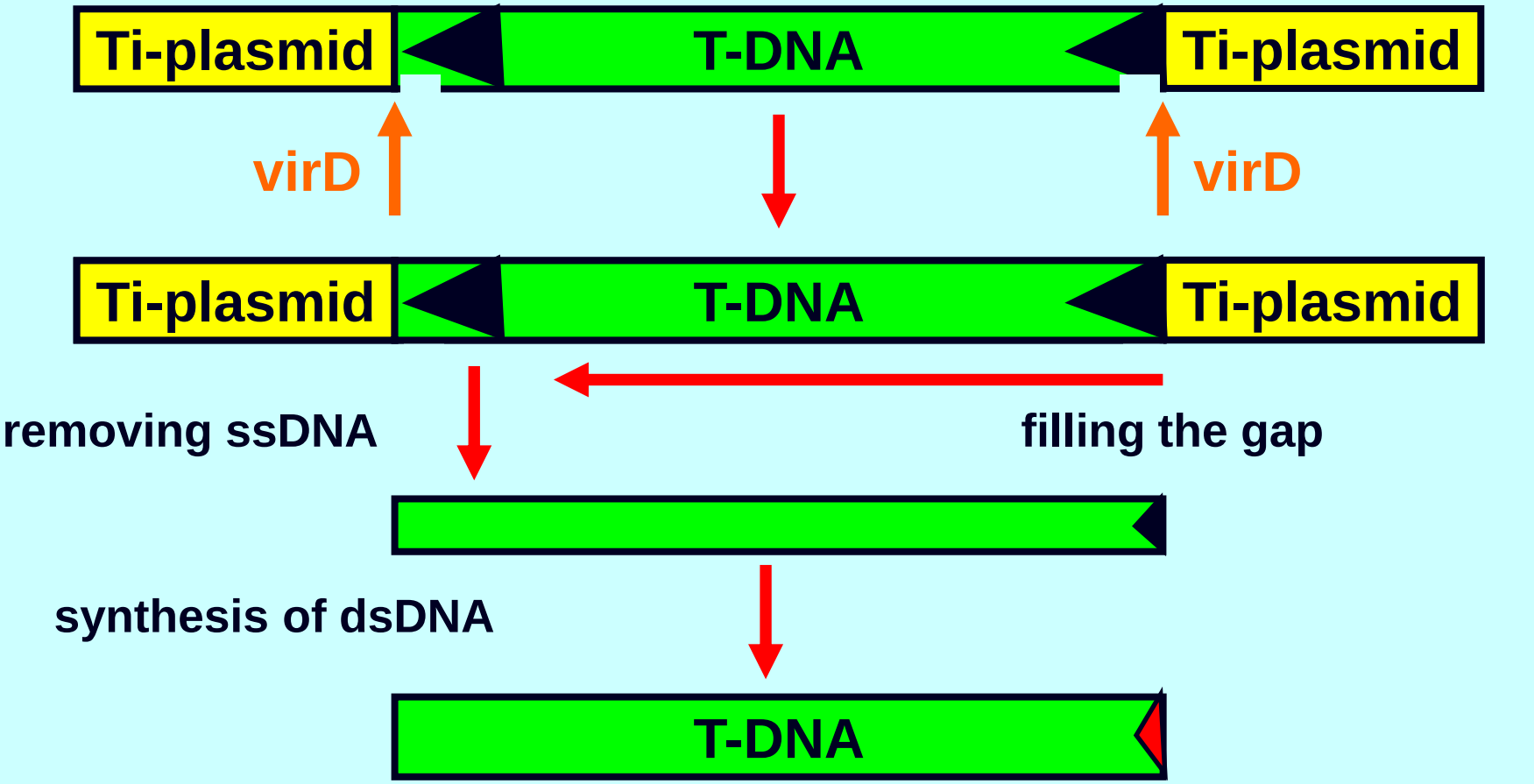
chemotaxis towards injured cells

injured plant cells



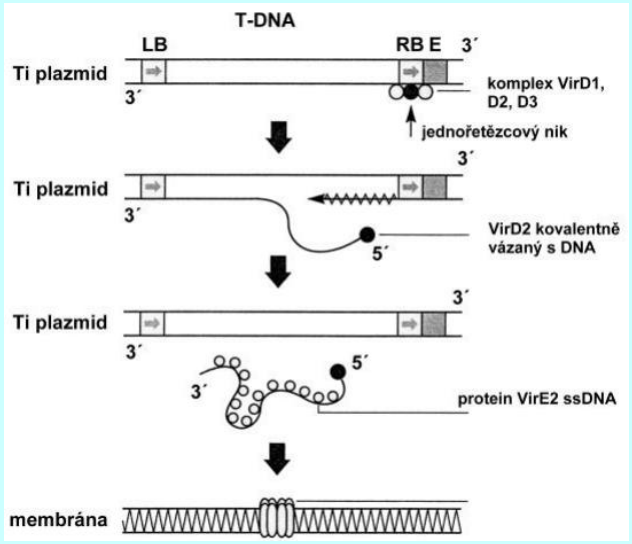
Function of the protein virD

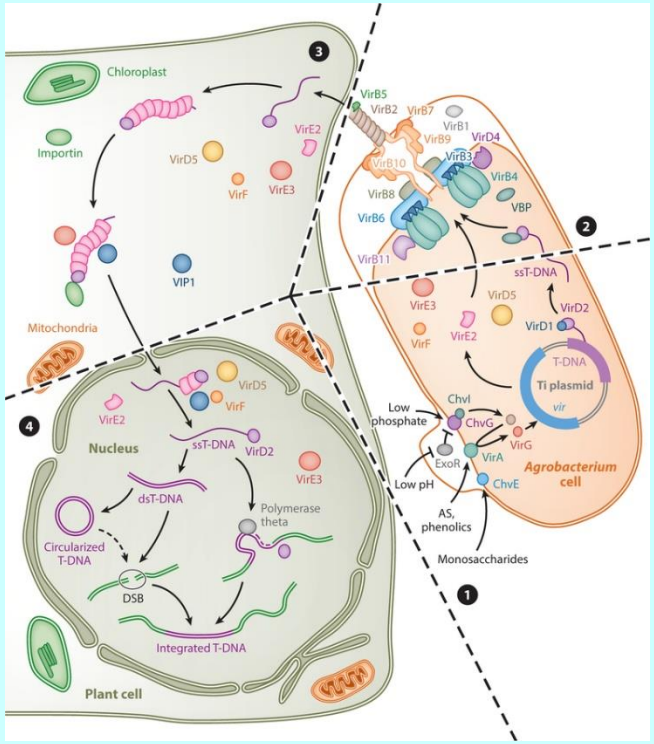
- Specific endonuclease
- Digests boundaries at the ends of T-DNA on Ti-plasmid

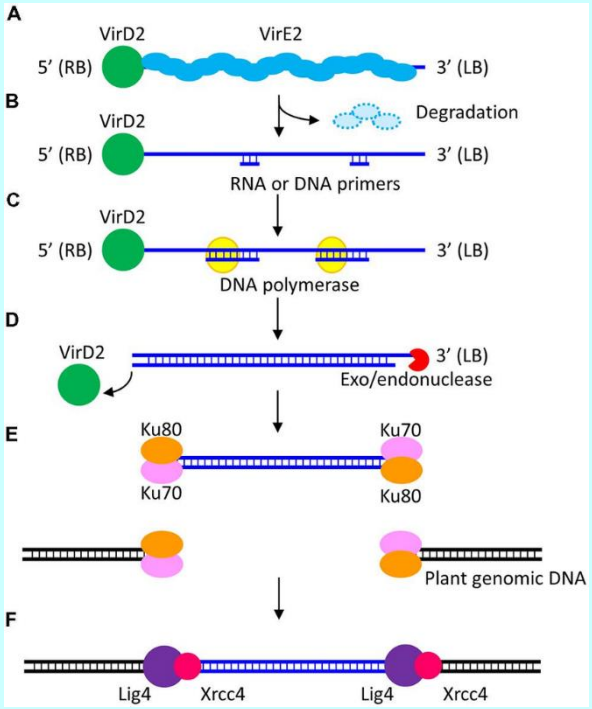


1 - 2 nucleotides of direct repetition

Transfer of T-DNA into plant







doi: 10.3389/fpls.2014.00560

DOI: 10.1146/annurev-phyto-082718-100101

Binary vectors with T-DNA

- **The Ti-plasmids themselves are too large → not easy to handle**
- **Use of binary vectors and "helper plasmids"**

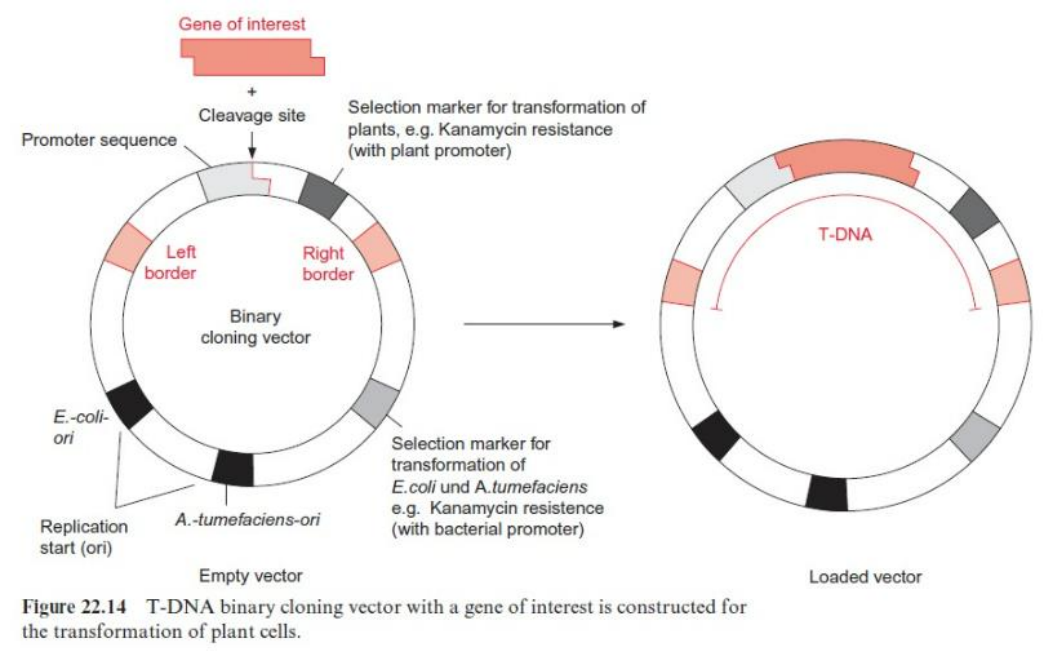
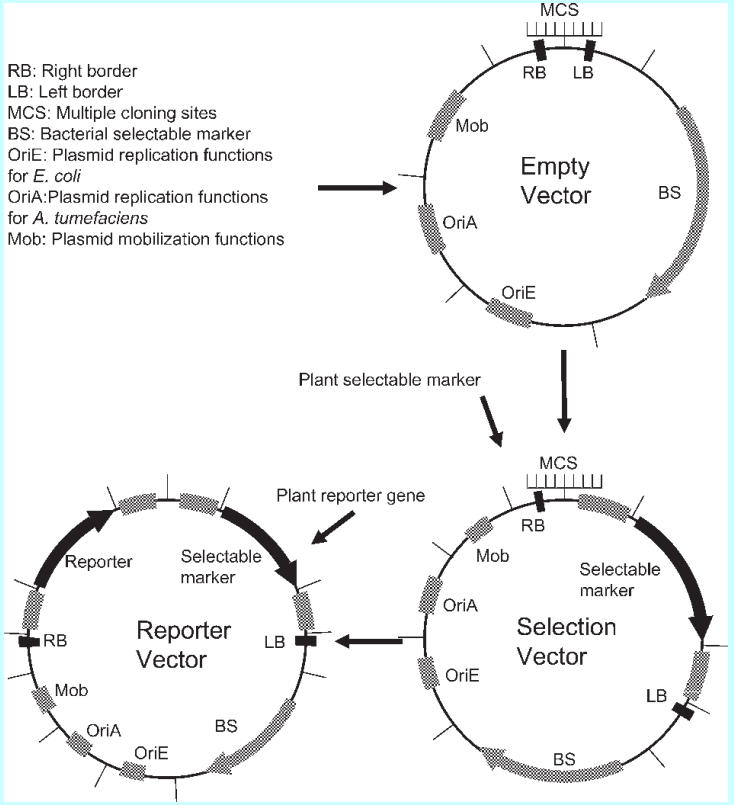


Figure 22.14 T-DNA binary cloning vector with a gene of interest is constructed for the transformation of plant cells.

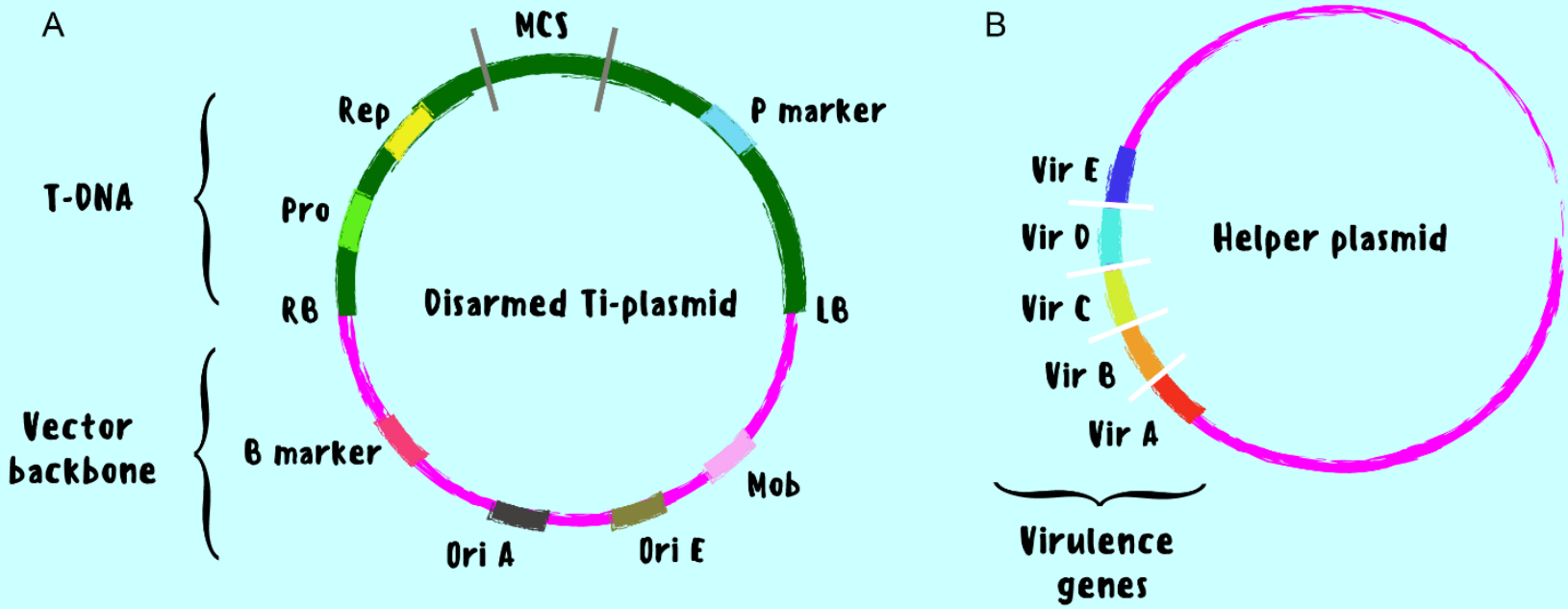
https://www.brainkart.com/article/Ti-Plasmids-are-used-as-transformation-vectors_21529/



DOI:10.1385/1-59745-130-4:15

Binary vectors + helper plasmids

- Binary vector (shuttle vector) – propagation in both, *E. coli* and *A. tumefaciens*
- „Helper plasmid“ – present directly in *A. tumefaciens*



The use of Ti and Ri plasmids

- **Modification and use of plasmid systems for the incorporation of cloned genes into the genome of crops**
- **Possibility to study gene functions in T-DNA, especially the genes for**
  - **other biosynthetic pathways of growth factors,**
  - **morphogenetic and physiological processes of plants (endogenous increase of the auxin and cytokine synthesis and resulting manifestations)**

Transformation of *Agrobacterium*

Scheme on the following slide

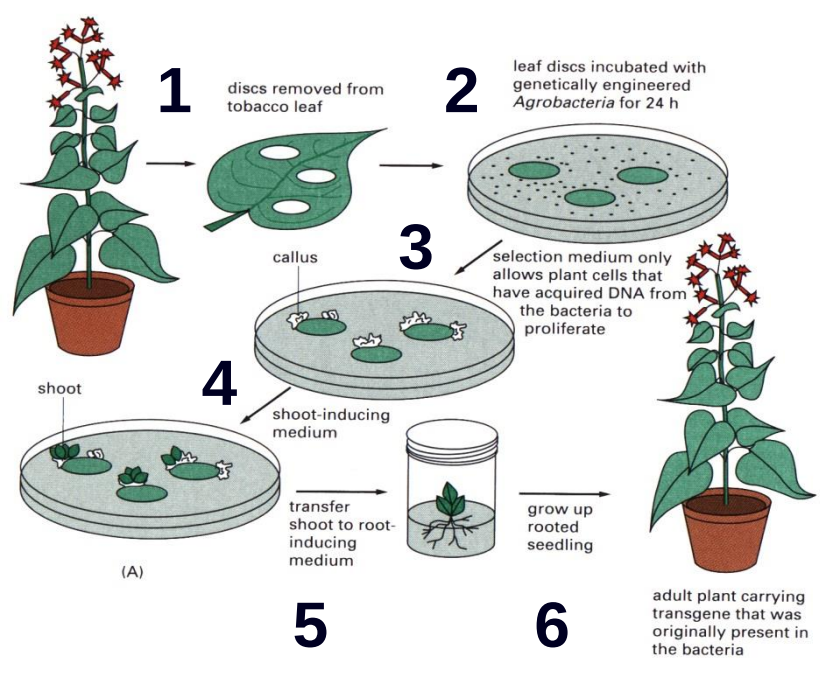
1) **Usually, infection of injured plant tissues *in vitro***

2) **Co-cultivation of leaf discs or suitable explantate**

3) **Screening the reporter genes expression**

4) **Regeneration of transformed cell and/or tissue into the whole plant**

Transformation of *Agrobacterium*



1) Extraction of leaf discs

2) Co-cultivation with GM *Agrobacterium*

3) Selection of transformants

4) Shoots induction

5) Roots induction

6) Regeneration of the whole plant

Alberts et al. (2002): Molecular Biology of the Cell, Figure 8-72

Characteristics of transformation by *Agrobacterium*

- **The insertions of T-DNA into the genome are occasional and their frequency is 1.5 times/transformation**
- **Concatemers are frequent results of insertions = consequence of promiscuous recombination**

Direct injection of DNA into a plant cell

- **DNA is transferred into cells but another transport into the nucleus and integration into the genome are not yet facilitated**
- **The method was developed for the transformation of monocotyledonous plants, which are not transformable by *Agrobacterium***

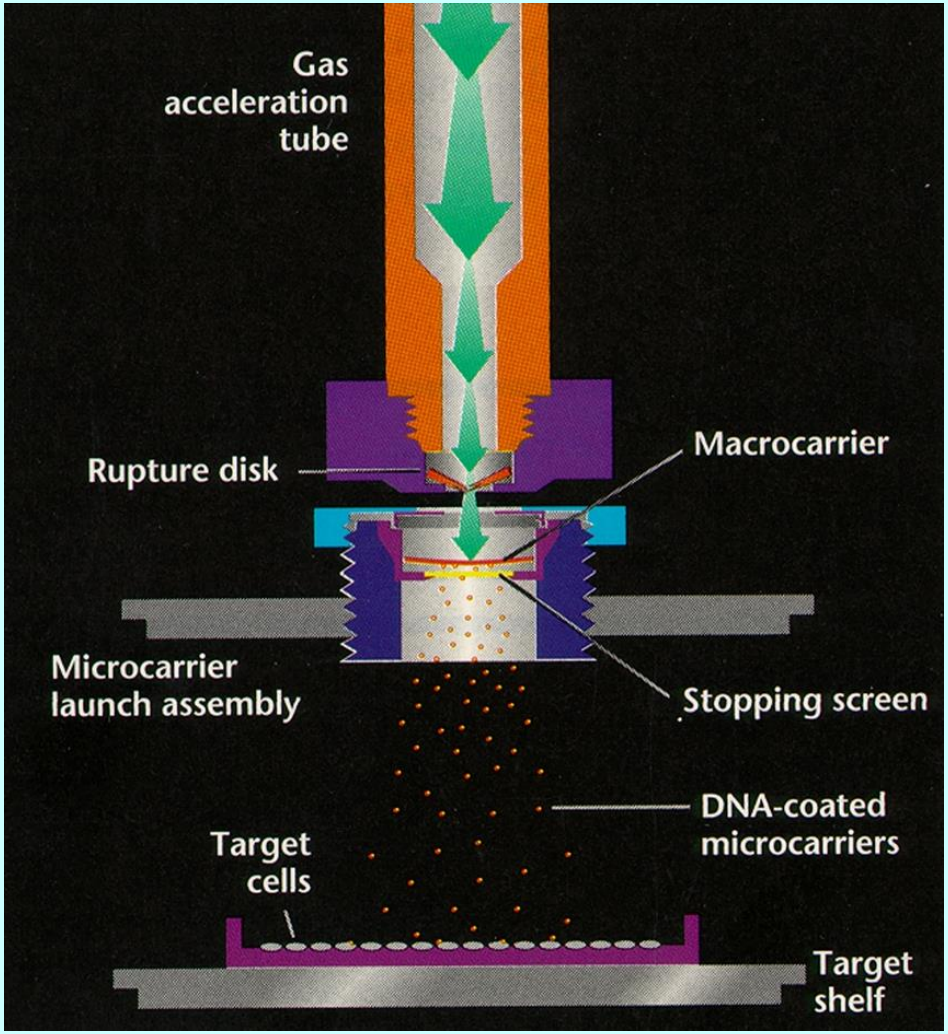
Types of direct injection of DNA

1) **Bombarding by microcarriers – gene gun**
2) **Protoplast transformation utilising polyethylenglycole or electroporation**

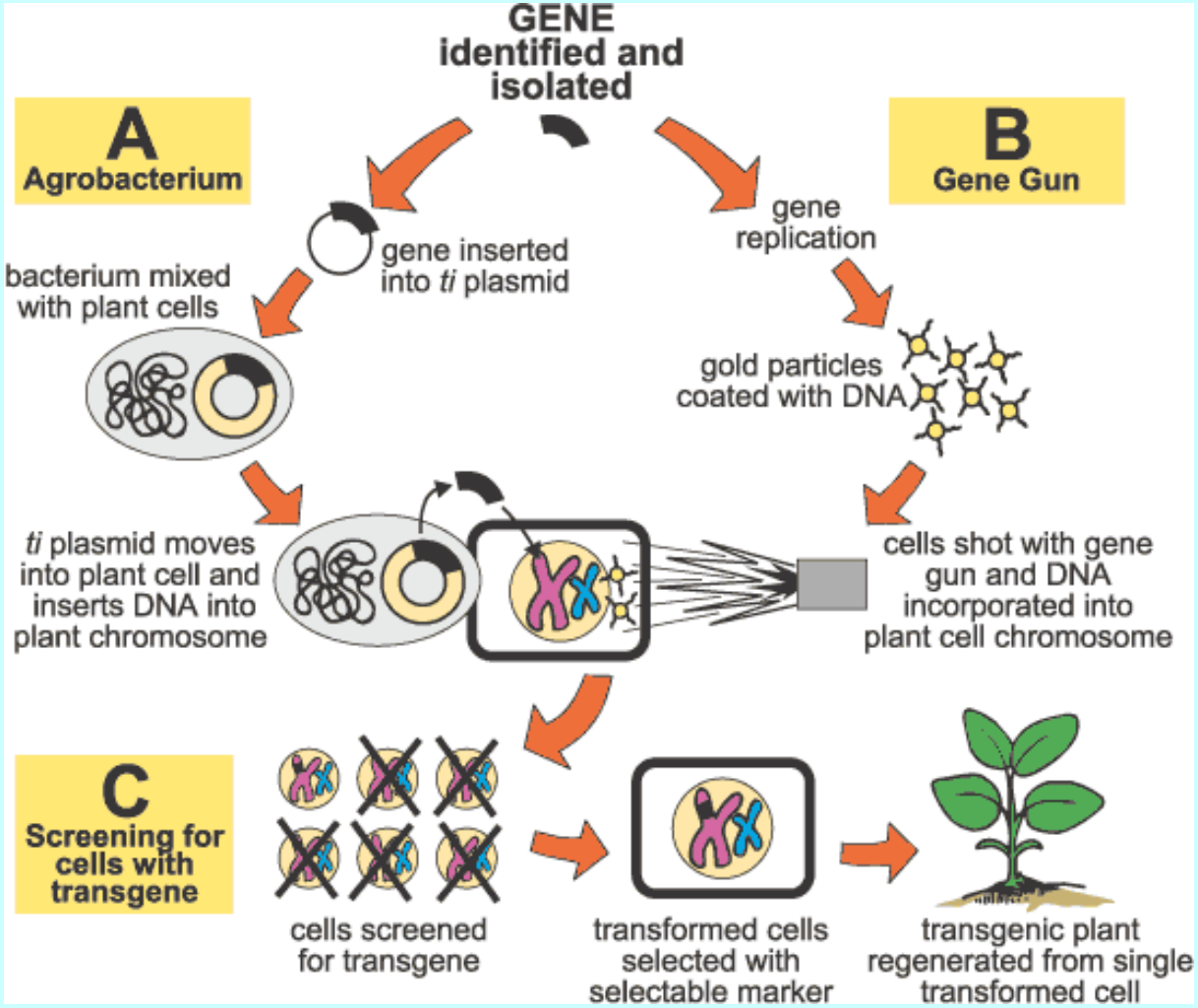
Bombarding by microcarriers (gene gun)

- **Gold or wolfram particles 1 - 2 μm in diameter, coated by DNA**
- **The particles bombard cells at high speed**
- **Plant cells remain vital, they are able to regenerate and divide by mitosis**
- **The process of transformation is influenced by several factors: size, number and speed of particles; type of DNA and its amount; amount, type and physiological status of cells**

Biolistic PDS-1000 system

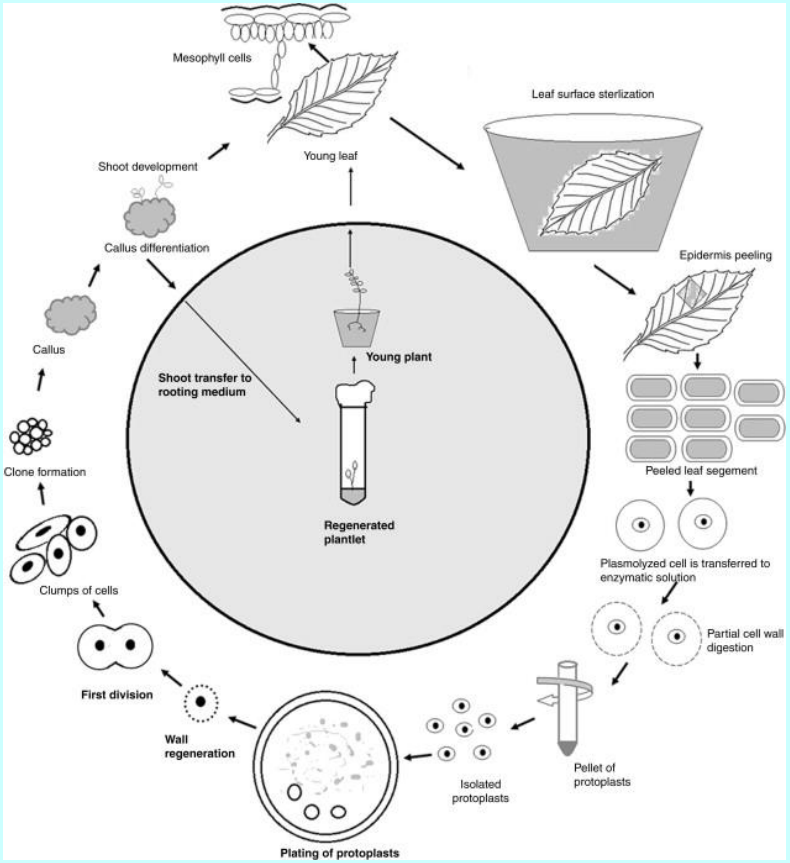


Comparison of the methods of transformation



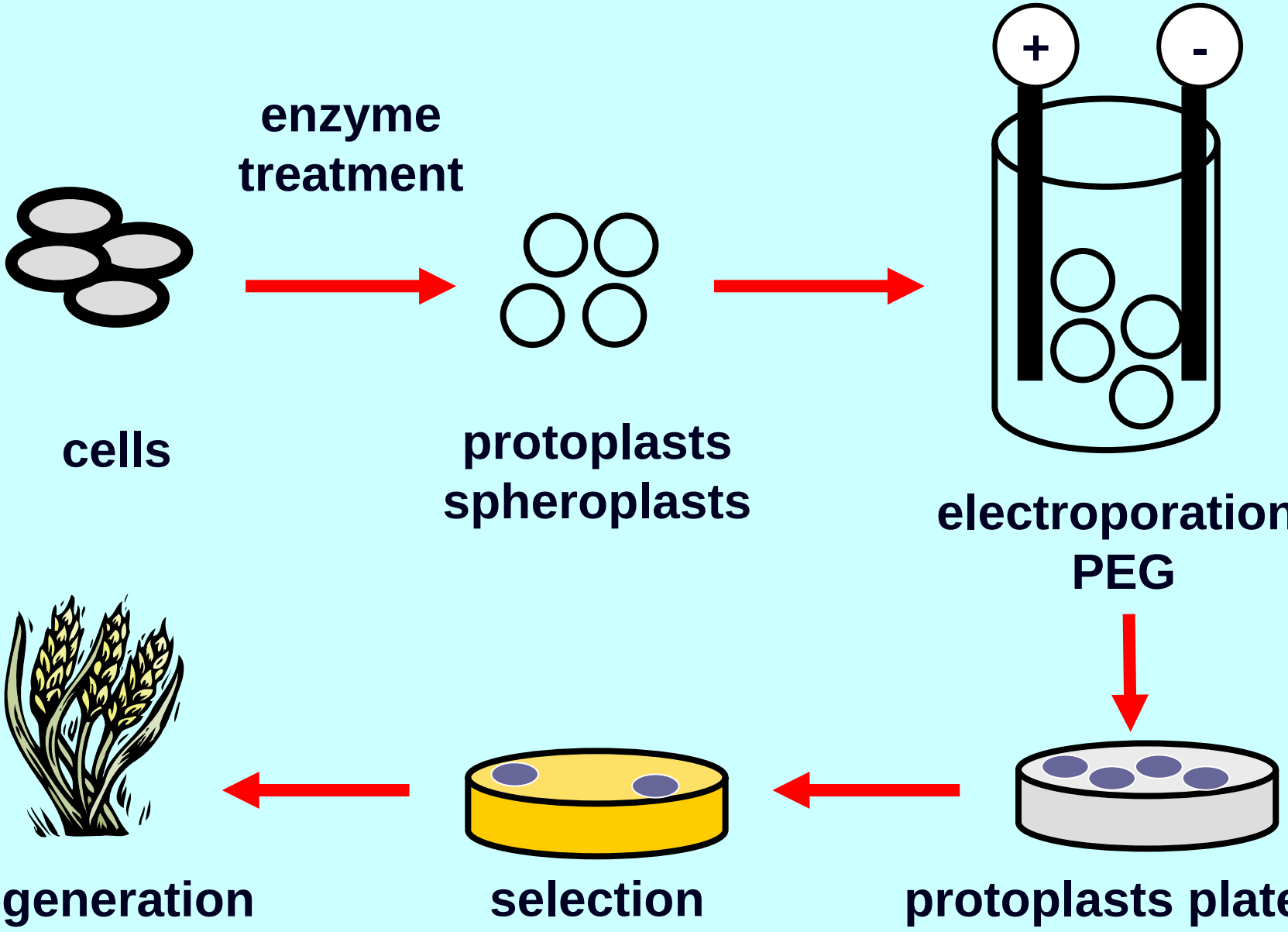
Protoplast transformation

- Similar principles of manipulation as animal cells
- Mainly used in monocotyledonous plants where *A. tumefaciens* cannot be used
- **Limitation -** difficult regeneration of an intact fertile plant from a protoplast



https://www.sciencedirect.com/topics/immunology-and-microbiology/protoplast-isolation

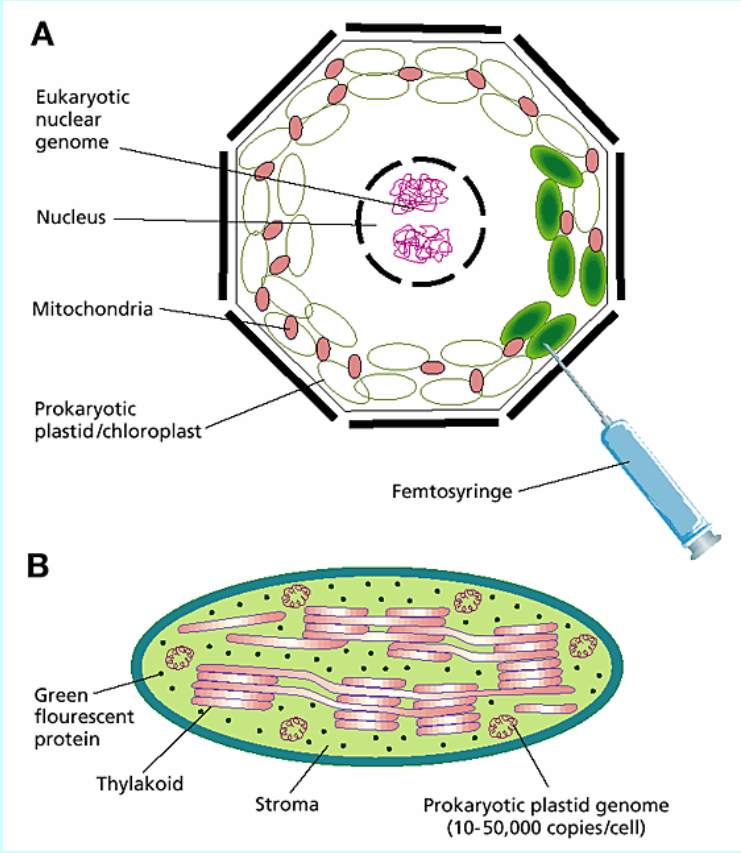
Protoplast transformation



Chloroplast transformation I

Benefits

- Elimination of pollen dispersal - plastids transmitted only through the maternal line
- Large number of cpDNA copies in chloroplasts (10-100 copies) → high expression of target protein (more than 30% of total soluble protein)
- Elimination of "gene silencing" - cpDNA does not contain compact chromatin structure



https://doi.org/10.1038/12841

Transformation methods

- "gene gun„
- direct femtoinjection

Chloroplast transformation II

- Integration of foreign DNA into cpDNA by homologous recombination
- Possibility of polycistronic mRNA expression

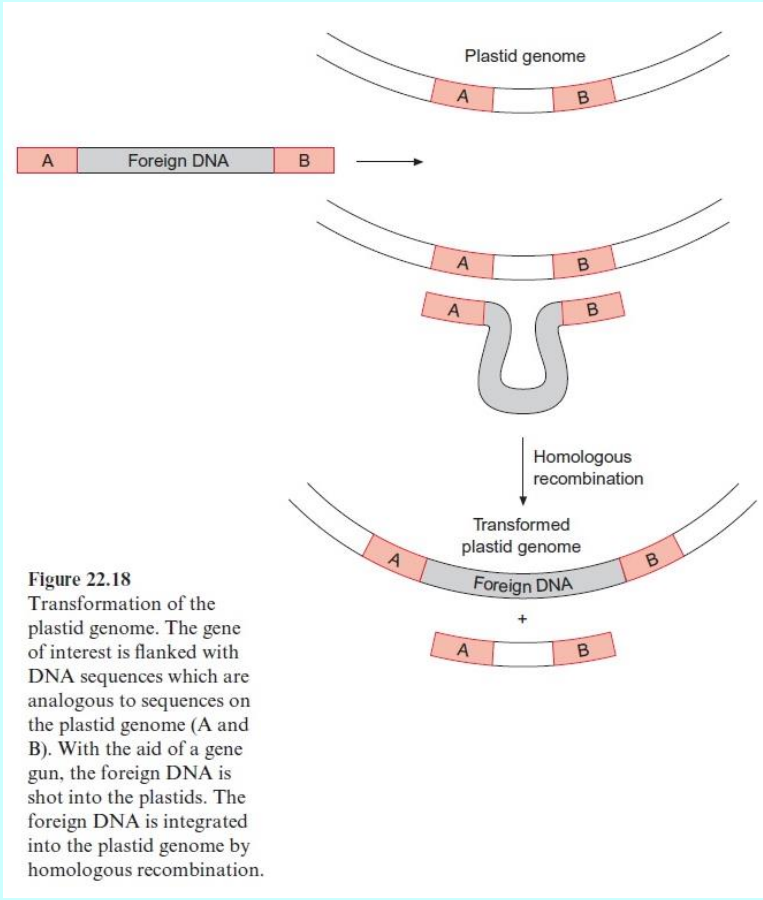


Figure 22.18
Transformation of the plastid genome. The gene of interest is flanked with DNA sequences which are analogous to sequences on the plastid genome (A and B). With the aid of a gene gun, the foreign DNA is shot into the plastids. The foreign DNA is integrated into the plastid genome by homologous recombination.

https://www.brainkart.com/article/Ti-Plasmids-are-used-as-transformation-vectors_21529/

Chloroplast transformation III

- Recently, the transformation of chloroplasts has gained importance. Transgenic plants obtained by transformation of the nuclear genome (e.g., by the Ti-plasmid) could pass their genetic information via pollen to cultivars in neighboring fields or in some cases also to related wild plants. This can lead to undesirable cross-breeding (e.g., causing the generation of herbicide-resistant weeds in the neighborhood of herbicide-resistant cultivars). This problem can be avoided when the genetic transformations are performed in the plastid genome of the plant. Since in most cases the plastid genome is maternally inherited, the genetic alteration will not spread to other plants via pollen transfer (pollination).

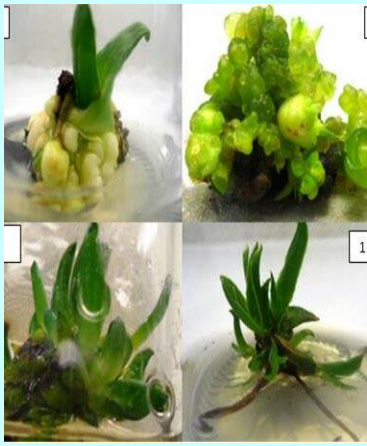
- The gene gun is usually used for the transformation of chloroplasts. The foreign DNA that is to be integrated into the plastid genome is provided at both ends with sequences, which are identical to sequences in the plastid genome (Fig. 22.18). After the foreign DNA has entered the plastids, it can be integrated into the plastid genome by homologous recombination at a site defined by the sequences at both ends. Whereas in the plant nuclear genome homologous recombinations are rare events, these occur frequently in the plastid genome. In this way random mutations are avoided, which occur when Ti-plasmids are used for transformation of the nuclear genome. A drawback of plastid transformation is, however, that plant cells contain many plastids, each with 10 to 100 genomes. By repetitive selection and regeneration, it is possible to achieve transgenic lines in which practically all the plastid genomes have integrated the foreign DNA (transplastome plants). Plants in which each cell contains many hundred copies of a foreign gene can be cultivated. This has the advantage that these transformed plants can produce large amounts of foreign proteins (up to 46% of the soluble protein), which might be relevant when the plants are to be used for the synthesis of defense compounds or pharmaceuticals.

https://www.brainkart.com/article/Ti-Plasmids-are-used-as-transformation-vectors_21529/

Once the recombinant plant is obtained, it needs to be further propagated and then the relevant metabolite needs to be extracted from it

Propagation most often occurs in controlled conditions called cell or tissue culture

Some words from history 1/2



The production of pharmaceuticals, cosmetics (and foods) derived from plant cell and tissue cultures has a long tradition

- 1902, Australian botanist Gottlieb Haberlandt - described the formation of a callus from an adult plant and its regeneration into a complete plant
- 1958, demonstrated totipotency of plant cells experimentally in vitro on carrot cells (Haberlandt)
- Between 1960s and 1980s - studies to mass propagate plant cell cultures and develop bioprocesses to deliver secondary metabolites

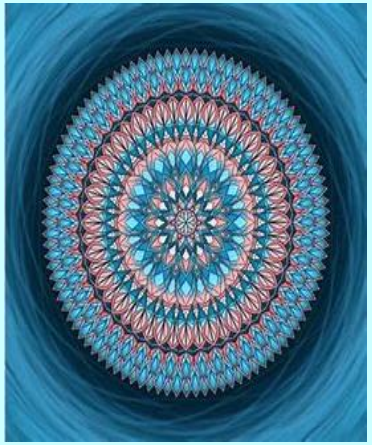
Some words from history 2/2

Pacific Yew cells grown in 75 m^3 stirred bioreactors deliver up to 500 kg of paxlitaxel per year

From the early 1980s to the late 1990s, various commercial secondary metabolites (e.g., shikonin, scopol amine, protoberins, rosmarinic acid, ginseng saponins, and immunostimulatory polysaccharides) based on plant cell cultures came on the market

2000, FDA approval of the anticancer compound paclitaxel

Benefits of cell culture



- **There is no seasonal dependence**
- **Controlled production through standardised batches is possible**
- **Low impact on the ecosystem - water demand and carbon footprint are reduced, and no pesticides or herbicide are needed**

- Nevertheless, the number of commercial secondary metabolite production processes involving plant cell cultures is low
- This is particularly true for pharmaceutical applications

Disadvantages of cell culture

- Somatoclonal variation of production clones
- Low titres of secondary metabolites

Which cells can be used?

1) Dedifferentiated callus cells (DDC)

2) Undifferentiated cambial meristematic cells (CMC)

- **All parts of the plant can be used, but**
- **It is important to select the most appropriate mother plant and organ type that contains the desired bioactive compound(s) in the required quantity and quality**
- **The quantity and quality of the bioactive compound of interest are greatly influenced by the species of plant, its developmental stage and the location and type of plant organ**

What else needs to be addressed?

We want a high-performance callus culture that is plump, grows and produces well, and is stable

- **Growth regulators (phytohormones that are a combination of auxins and cytokinins)**
- **Nitrogen, phosphate and sucrose levels**
- **The type and concentration of growth regulators affect growth and callus morphology as well as the synthesis of secondary metabolites**

Murashige and Skoog 1962

Establishing a DDC-based explant culture

Selection of starting material

Surface sterilisation

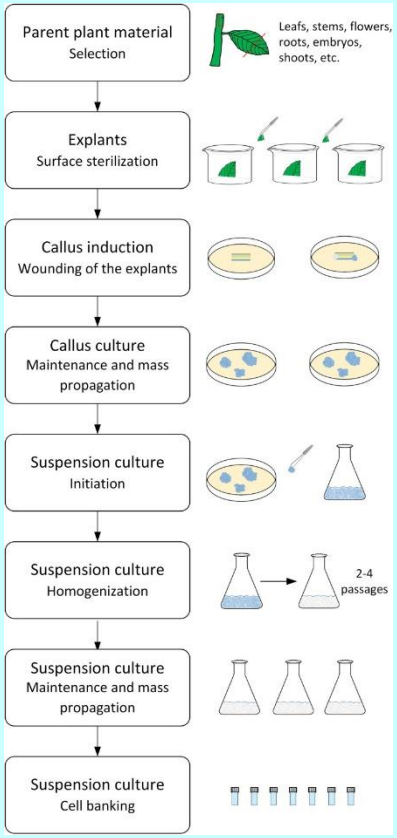
Callus induction

Cultivation of callus

Establishment of suspension culture

Homogenization of suspension culture

Maintenance of suspension culture



How do DDCs grow?

- Doubling time between 2 and 4 days
- Grow in aggregates of up to hundreds of cells
- Formation of aggregates = formation of extracellular polysaccharides, can affect cell growth and product formation
- Genetic instability due to somaclonal variation may occur with increasing culture time

- Cryopreservation, which, unlike mammalian cells, is more complicated - more difficult to regrow after thawing

Are CMCs better?

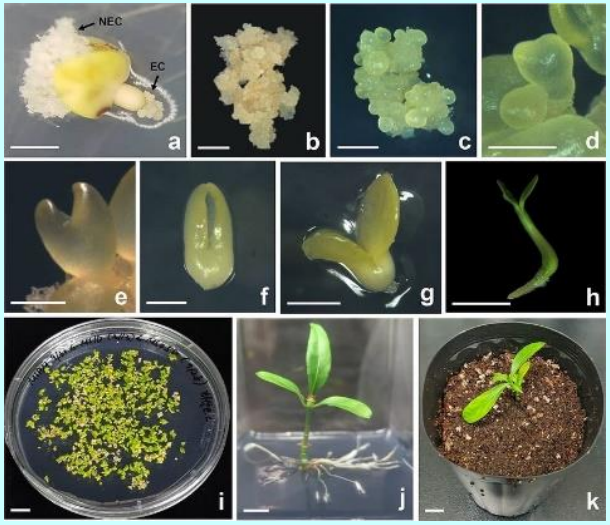
- **Undifferentiated cambial meristematic cells have spherical abundant vacuoles, are morphologically and physiologically stable, grow as single cells and are easily recovered after cryopreservation**
- **No need for homogenisation**

- **Unhwa, owner of the world's first patent for CMC isolation and cultivation, has successfully developed suspension cultures based on the CMCs *Taxus cuspidata*, *Ginkgo biloba* and *Solanum lycopersicum* for applications in the cosmetics industry**

Tissue cultures

- **They are of minor importance in the cosmetic industry**

  - **Products based on hair roots (e.g. RootBioTec HO from *Ocimum basilicum* by Mibelle Biochemistry)**
  - **Somatic embryo culture products (e.g. Vita Nova from *Lotus japonica* by Vitalab).**



Cultures derived from hair roots

- Characterized by lateral branching, similar growth to suspension cultures of plants
- Reproduce without hormones
- They do not exhibit geotropism
- They are genetically stable

Eibl, R. et al. (2018): Applied Microbiology and Biotechnology (2018) 102:8661–8675

https://doi.org/10.1007/s00253-018-9279-

However, they can only be used to produce bioactive compounds synthesized in the roots of the parent plant

Cultures derived from embryonic cells

- Morphologically and physiologically identical to zygotic embryos present in the seeds of the mother plant
- Induced by either differentiated or undifferentiated somatic cells through a series of morphological and biochemical changes

Eibl, R. et al. (2018): Applied Microbiology and Biotechnology (2018) 102:8661–8675

https://doi.org/10.1007/s00253-018-9279-

Culture of somatic embryos at the end of somatic embryogenesis, so-called torpedo stage embryos

Summary

1. Structure of plant DNA
2. What can be manipulated in plants
3. Genetic transformation of plants
4. Transformation by *Agrobacterium*
5. Ti/Ri plasmids, T-DNA
6. Transformation by “naked“ DNA
7. Protoplast transformation
8. Plant tissue cultures